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Role of citrus volatiles in host recognition, germination and growth of *Penicillium digitatum* and *Penicillium italicum*

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Abstract

Volatiles emitted from wounded peel tissue of various citrus cultivars had a pronounced stimulatory effect on germination and germ tube elongation of both *Penicillium digitatum* and *P. italicum*; however, *P. digitatum* appeared to be more sensitive to the stimulatory action of citrus peel volatiles. When exposed to volatiles from grapefruit peel discs, the percentage of germinated spores of *P. digitatum* and *P. italicum* was 75.1% and 37.5%, respectively, whereas germination of controls was 6.8% and 14.7%, respectively. In contrast, *Botrytis cinerea* and *P. expansum* were either not affected or inhibited by the peel volatiles. GS–MS analysis of volatiles present in the peel of various citrus fruit cultivars revealed that limonene is the major fruit peel volatile. Its percentage ranged from 89% to 95% at the early stages of fruit development throughout the harvest season. Myrcene and α-pinene made up the second and third greatest amounts among the volatiles found in these oils, ranging from 2.12% to 2.33% and from 0.71% to 1.25%, respectively. All four monoterpenes, limonene, α-pinene, β-pinene and myrcene were stimulatory to *P. digitatum* and *P. italicum* but inhibitory to or had no effect on *P. expansum* and *B. cinerea*. Germ tube elongation in *P. digitatum* responded most strongly to limonene and less strongly to α-pinene and β-pinene while myrcene had little effect. In contrast in *P. italicum*, myrcene stimulated germ tube elongation the most followed by limonene, with α-pinene, and β-pinene being about equal. Germination of *P. italicum* conidia was highest in response to myrecene with the effect of the other compounds being about equal at concentrations of 5 μL or more per plate.

Keywords: Citrus; Volatiles; Limonene; Spore germination; Hyphal growth promotion

1. Introduction

Green and blue molds, caused by *Penicillium digitatum* Sacc. and *P. italicum* Wehmer, respectively, are the most damaging postharvest diseases of citrus fruit. These two *Penicillium* species do not cause postharvest decay in any other fresh fruit or vegetable crops. The etiology of these diseases is well understood. Dormant *Penicillium* spores present on the fruit's surface become active if the peel is injured. The spores germinate rapidly and colonize the injured tissue. The initial symptom, a softening of the exocarp, is visible within 48 h. In theory, if fruit injury could be reduced to zero, no disease would be present. However, this is impractical and disease control based solely on gentle handling has never succeeded. Hence, citrus producers are heavily reliant upon fungicides for decay control. While the etiology of *Penicillium* rots is well understood, the physiological and biochemical basis of its host specificity is much less clear. Unlike the spores of many fungi, conidia of *P. digitatum* and *P. italicum* do not germinate on a medium lacking a carbon source such as water agar or on the surfaces of intact

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fruit (Kavanagh and Wood, 1971; Eckert et al., 1984), but do germinate if exposed to volatiles released in injured citrus fruit. Both volatile and non-volatile components of citrus peel have been shown to stimulate germination and growth of *P. digitatum* and *P. italicum*. Ethanol extracts of grapefruit peel were found to stimulate the growth of *P. digitatum* and *P. italicum* but had little effect on *P. expansum*—a non-pathogen of citrus (Stange et al., 2002). Further studies indicated that specific components of this extract were responsible for stimulation. Knowledge on the physiological mechanism of perception and the biochemical nature of spore germination following exposure to stimulatory volatiles released from wounds is based on only two conflicting reports by French et al. (1978) and Eckert and Ratnayake (1994) in which different mixtures of variety of volatiles were suggested to be responsible for stimulation of spore germination.

Kavanagh and Wood (1971) found that conidia of *P. digitatum* placed on water agar plugs on the surface of intact oranges did not germinate, but if plugs were placed over puncture wounds in the peel germination was >80%. Peel oils from several species of citrus and volatiles emitted from injured oranges were reported to be responsible for stimulating germination of *P. digitatum* conidia (French et al., 1978; Eckert et al., 1984). Of ten *Penicillium* species tested, only *P. digitatum* and *P. italicum* spores displayed enhanced germination on water agar when exposed to volatiles from injured oranges (Eckert et al., 1992). In studies using amended water agar, germination of *P. italicum* was stimulated by citral, nonanal, citronella, and citronellal, but *P. digitatum* was stimulated only by citral (500 μL/L); this stimulation was further enhanced if nonanal (500 μL/L) was added (French et al., 1978). Combinations of limonene, acetaldehyde, ethanol and carbon dioxide stimulated spore germination of *P. digitatum*, although individual components alone were inactive (Eckert and Ratnayake, 1984). A comprehensive understanding of the involvement of volatiles in *Penicillium* spore germination is further complicated by their inhibitory effects when assayed using rich growth media, rather than water agar. Aliphatic aldehydes (C 5–9) were found to inhibit germination of *P. digitatum* conidia (Davis and Smoot, 1972). Octanal was the most potent, with an IC-50 of 60 μL/L. Citral has been shown to reduce germ tube elongation of *P. digitatum*, with an IC-50 of ∼100 μL/L (Rodov et al., 1995). Based on reports in the literature, both stimulatory and inhibitory affects have been attributed to citrus volatiles, particularly citral, octanal and nonanal.

The paucity of information on stimulatory volatile compounds in citrus peel is in striking contrast to the numerous studies on the antifungal properties of induced and constitutive compounds from citrus fruit (Stange et al., 1993; Rodov et al., 1995; Vargas et al., 1999). In the present work, we describe the stimulatory effect of wound volatiles of different citrus cultivars on spore germination and germ tube elongation of *P. digitatum* and *P. italicum* as compared to their effects on two other common postharvest pathogens, *P. expansum* and *B. cinerea*, that are not pathogenic on citrus fruit. In addition, the composition of wound headspace and citrus oil volatiles of three citrus cultivars were characterized using GC–MS and the effect of major components of the volatiles on both pathogenic and non-pathogenic fungi were determined. The results obtained provide evidence that the stimulation of spore germination and growth of *P. digitatum* and *P. italicum* by the major, specific components of wound volatiles is concentration dependent.

2. Materials and methods

2.1. Fruit materials

'Star Ruby' red grapefruit (*Citrus paradise* Macf), 'Valencia' oranges (*Citrus sinesis*), lemon (*Citrus limons* L.) and clementine (*Citrus reticulata* L.) were obtained at different stages of harvest maturity (August 2004–March 2005) from a commercial orchard in Bet Dagan, Israel. Fruit were used immediately after harvest or stored at an optimal storage temperature for each fruit (grapefruit and lemons 9–10 °C, clementines and oranges, 5 °C) until used. Pear, apple, tomato, pepper, strawberry and avocado fruit were purchased from a local market.

2.2. Chemicals

Synthetic compounds used in the bioassays and as references for identification of the citrus volatiles were: α-pinene (98%), α-terpineol (97%) β-pinene (99%), citral (cis + trans) (95.0%), limonene (97%), nonanal (95.0%), octanal (98.0%) all supplied by Sigma–Aldrich Ltd., Steinheim, Germany. Myrcene, linalool, orange oil, mandarine oil, and grapefruit oil were supplied by Frutarom Co., Haifa, Israel.

2.3. Fungal cultures and preparation of the inoculum

Strains of *P. digitatum*, *P. italicum*, *P. expansum* and *B. cinerea*, were obtained from the culture collection of the Department of Postharvest Science, ARO, the Volcani Center. Spore suspensions were prepared from 2 to 3 week old PDA (Potato dextrose Agar, Difco, Detroit, MI) cultures of the pathogens. Spores were removed from the edges of the sporulating cultures with a sterile disposable plastic bacteriological loop (Miniplast, Ein-Shemer, Israel), and suspended in sterile distilled water. Mycelial fragments remaining were removed by filtration through 4 layers of sterile cheesecloth. Spores were washed twice with sterile distilled water to remove any remaining nutrients. Spores were pelleted by centrifugation at $3000 \times g$ for 5 min and resuspended in sterile distilled water. The concentration of the suspension was adjusted to 8×10^4 spores mL^{-1} using a hemacytometer.

2.4. Effect of citrus volatiles on spore germination

A Petri dish bioassay system was developed to asses the effect of various volatiles (peel discs, pure compounds and crude citrus oils) on spore germination and germ tube development of the various pathogens. Three separate 15 μL drops of a spore suspension (8×10^4 spores mL^{-1}) of a specific pathogen was placed in different locations on the surface of the 3% sterile water agar (Difco, Detriot, MI). Each spot served

as one replication. After the drops were absorbed into the agar, the plates were turned over and a 13 mm filter disk (Whatman paper) was placed on the cover of each of the plates. Increasing concentrations of citrus crude oils (5, 10, 20, 40 μL per plate) or pure volatile compounds (0.005–60 μL per plate) were applied to each filter disk in order to assess the effect of volatiles emitted from fruit, two peel discs (15 mm in diameter) were cut with cork borer and placed on the cover of each plate instead of a filter disk. Control plates contained paper disks without any substance. The plates were sealed with PVC tape and incubated at 25 °C for 24 h. Fungal spores were then stained by adding 10 μL of lactophenol blue (Sigma–Aldrich Ltd.) to each replication on the plate and viewed using a Leica microscope. Images were acquired using a Leica color digital camera (Leica DC200). Percentage of germination and average germ tube length of the pathogens were determined in three microscopic fields containing at least 30 spores each and analyzed by Leica IM 1000 software. Mean values of the three replications and standard errors were calculated. An approximate calculation of the volatile concentration (μL/mL) applied in plates was made for comparative purposes. The approximate concentration was calculated as the amount of the volatile compound applied to the paper disc divided by the void volume of the Petri dish.

2.5. Composition of crude citrus oils

Crude citrus oils (20 μL/mL) were diluted in methyl-*tert*-butyl ether (MTBE) prior to injection. Samples were analyzed using an Agilent gas chromatograph (6890N) equipped with a 30 m × 0.25 mm (d_f = 0.25 μm) silica gel capillary column (Rts—5 Sil MS), a flame ionization detector (FID) and an Agilent (6890N) mass spectrometer. Injector and detector temperatures were 250 °C and 330 °C, respectively. Injection volume was 1 μL. For identification, the GC retention index was compared to authentic standards and with the HPCH1607 GC–MS library. For quantification, the peak areas from three injections were used to calculate the concentrations and standard errors. For routine analysis, outputs of the FID alone were used.

2.6. Analysis of volatiles in the peel and in the dynamic headspace of injured fruit

Two methods were used to investigate citrus volatiles. The first method, peel extraction, was used to determine what compounds were present in the peel. The second, dynamic headspace analysis, was used to determine what volatile compounds were present in the atmosphere in close proximity to the fruit surface. To determine the composition of volatile compounds in citrus peel, three flavedo discs (15 mm in diameter) were cut with a cork borer from each type of fruit, weighed and then immersed and shaken for 1 h in 5 mL of MTBE containing 10 μg ethyl myristate as an internal standard. Three replicate samples consisting of three discs per sample were analyzed for each type of fruit. Each sample was dried with Na_2SO_4 and concentrated under a gentle stream of nitrogen to 0.5 mL. One μL of each sample was analyzed by GC–MS as already described.

Volatiles in the dynamic headspace of wounded fruit were determined as follows. An incision, 4 cm long and 2 mm deep was made in peel of fruit using a scalpel. Each fruit was placed on a glass surface, covered with a 150 mL glass funnel, and its edges sealed to the glass plate with PVC tape. To absorb wound volatiles, a glass column (6 cm × 0.5 cm) containing Porapak Q (Sigma) powder was connected to the funnel tube. The column was connected via a silicone tubing (0.5 m × 0.5 cm) to a vacuum pump for 24 h. The glass column was then disconnected from the system and the Porapak Q powder was extracted by washing it with 10 mL of hexane. One ml of MTBE containing 10 μg/mL of ethyl myristate was added to the sample as an internal standard. Each sample was dried with Na_2SO_4 and concentrated under a gentle stream of nitrogen to 0.5 mL. Samples were analyzed by GC–MS.

2.7. Fluorescence microscopy

Lemon fruit were thoroughly washed with tap water, dipped in hypochlorite solution (0.05%) for 1 min to disinfect the peel surface, and then rinsed with sterile water. A spore suspension of *P. digitatum* was prepared from 2 week old PDA *P. digitatum* culture and adjusted to 10^5 spores per mL as already described. Oil glands in an area of 5 mm × 5 mm on the surface of the fruit were punctured using a sterilized microbiological needle. Three to four 5 μL drops of spore suspension were placed at a distance of ~5 mm around each wounded area. Droplets of spores placed on intact fruit surfaces were used as controls. Fruit were sealed in 1 L glass jars and incubated at 25 °C. At 40 and 140 h after inoculation peel slices (1 cm × 1 cm, 1–2-mm thick) containing the wounded area were cut off with a scalpel and, fixed and stained with calcofluor brightener according to Rohringer et al. (1977). Samples were analyzed with a Leica microscope equipped with fluorescence system and images were acquired using a Leica color digital camera (Leica DC200).

3. Results

3.1. Effect of fruit volatiles on germination of P. digitatum and P. italicum

The effect of volatile compounds emitted from rind tissue of different citrus cultivars, pear, apple, tomato, pepper, strawberry and avocado fruit on spore germination and germ tube elongation of *P. digitatum* and *P. italicum* is presented in Fig. 1. Volatiles emitted from peel tissue of all citrus cultivars tested had a pronounced stimulatory effect on germination and germ tube elongation of both *P. digitatum* and *P. italicum*; however, *P. digitatum* appeared to be more sensitive to the stimulatory action of citrus peel volatiles. When exposed to volatiles from grapefruit peel discs, the percentage of germinated spores of *P. digitatum* and *P. italicum* was 75.1% and 37.5%, respectively, whereas germination of controls was 6.8% and 14.7%, respectively. However, germination and growth of spores of both fungi exposed to discs of other fruit was not affected (data from tests with *P. italicum* is not shown) (Fig. 1).

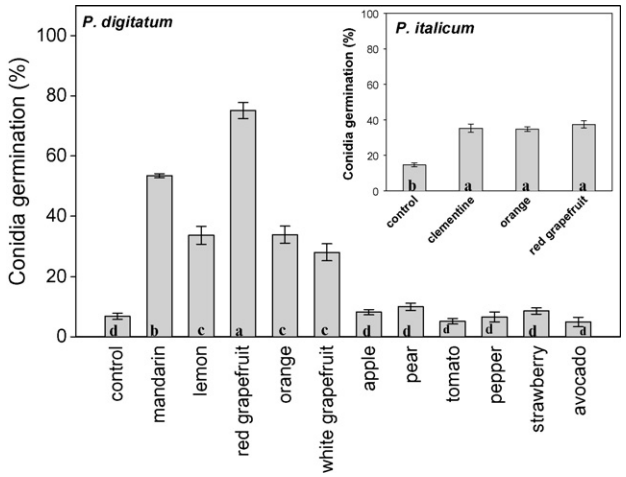

Fig. 1. Effect of volatiles released from peel discs of different fruit on germination and growth of *P. digitatum* and *P. italicum*. Mean ± S.E. in three microscopic fields containing at least 30 spores each. Treatments with different letters are significantly different at $P \leq 0.05$.

3.2. Bioassay of the effect of citrus fruit peel volatiles on germination of P. digitatum, P. italicum, B. cinerea and P. expansum

The effect of citrus fruit volatiles on germination of *B. cinerea* and *P. expansum*, non-citrus pathogens, compared with that of *P. digitatum* and *P. italicum* was determined. The bioassay using fruit discs was performed with clementine, Valencia orange and Ruby Red grapefruit. Results presented in Fig. 2 indicated that volatiles emitted from peel tissue of different citrus fruit cultivars had a pronounced stimulatory effect on spore germination of both *P. digitatum* and *P. italicum* but the degree of stimulatory effect was much greater on *P. digitatum* compared to *P. italicum*. In contrast, *B. cinerea* and *P. expansum* were either not affected or inhibited by the peel volatiles. *P. digitatum* exhibited a maximum 6.5-fold increase in germination compared to controls using clementine fruit peel discs while *P. italicum* exhibited a 2.5–3-fold increase in germination compared to controls using Valencia orange and grapefruit fruit peel discs, respectively. On the other hand, germination of *B. cinerea* and *P. expansum* was either not affected or inhibited by volatiles released from fruit peel discs of all three different citrus fruit cultivars.

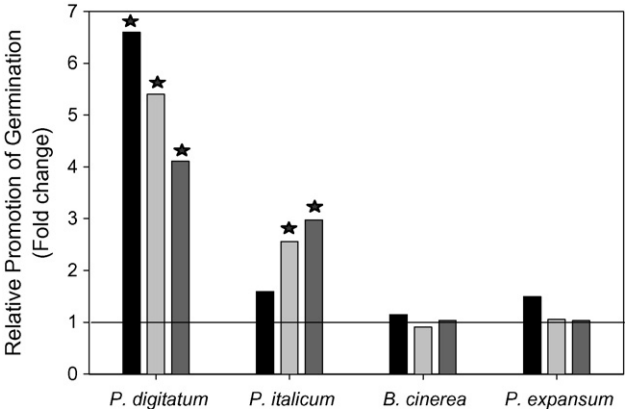

Fig. 2. The effect of volatiles released from peel discs of clementine ( ) orange ( ) and grapefruit ( ) germination of *P. digitatum*, *P. italicum*, *B. cinerea* and *P. expansum*. Values represent the fold increase in germination compared to control samples. Values above and below the line indicate promotion or inhibition of germination, respectively. Columns with asterisk indicated mean separation from the control as determined using a *t*-test ($P \leq 0.05$).

3.3. Effect of commercial citrus oil volatiles on germination and growth of P. digitatum, P. italicum, P. expansum and B. cinerea

The effect of oil volatiles of mandarin, grapefruit and orange fruit on germination and growth of *P. digitatum*, *P. italicum*, *P. expansum* and *B. cinerea* is presented in Fig. 3. *P. digitatum* and *P. italicum* exhibited enhanced germination and a stimulation of germ tube growth following exposure to all oils tested. *P. digitatum* had the highest percentage of germination (88.4%) observed in Petri dishes supplemented with 5 μL mandarin oil, compared to controls (2.9%). A similar stimulation of germination and germ tube growth was also observed with *P. italicum*. The effect of the volatiles was concentration dependent and at 20 and 40 μL/plate a decrease in the stimulatory effect of the oils was observed depending on the pathogen/oil combination.

P. expansum and *B. cinerea* responded differently to exposure to citrus oil volatiles and in contrast to the stimulatory effect observed with *P. digitatum* and *P. italicum* a marked inhibitory effect on both spore germination and germ tube elongation was evident. Percent inhibition of *P. expansum* spore germination at 20 μL/plate of mandarin and orange oils was 70% and 94%, respectively. Spore germination was completely inhibited at 40 μL/plate. *B. cinerea* was less sensitive to mandarin and orange oils and significant inhibition was achieved only at 40 μL/plate. In contrast, grapefruit oil was less inhibitory to *P. expansum*, whereas, *B. cinerea* was completely inhibited at 20 and 40 μL grapefruit oil (Fig. 3). GC–MS analysis of the compounds applied to the paper discs was conducted to determine the actual amount of volatiles released to the headspace. Results indicated that more than 95% of the amount of various volatiles applied to the disc was released to the headspace within 6–8 h (data not shown).

3.4. Identification and quantification of volatile constituents of the commercial mandarin, orange, and grapefruit oil

To determine if the stimulatory effect observed in host-specific citrus pathogens and inhibition in non-host pathogens was due to single or multiple volatile constituents, the citrus oil from mandarin, grapefruit and orange peel was fractioned by GC and its molecular constituents identified by mass spectrometry (Table 1). Analysis of mandarin, orange, and grapefruit oil showed that limonene was the most abundant constituent, making up 88.9%, 86.8%, and 92.34% of the total volatiles in mandarin, orange, and grapefruit oil, respectively. Myrcene and α-pinene made up the second and third greatest amounts among the volatiles found in these oils, ranging from 2.12%

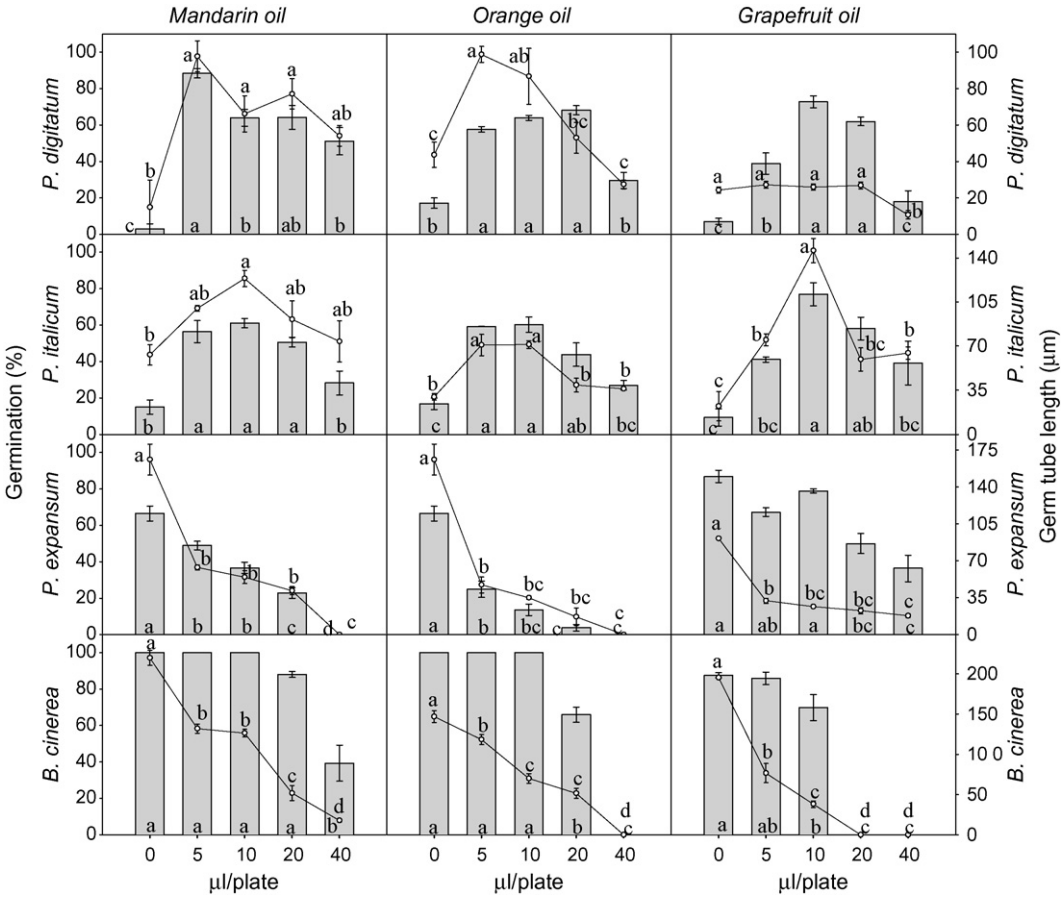

Fig. 3. Effect of commercial citrus oils on percent germination ( ) and growth (-◯-) of *P. digitatum*, *P. italicum*, *P. expansum* and *B. cinerea*. Mean ± S.E. in three microscopic fields containing at least 30 spores each. Treatments with different letters are significantly different at $P \leq 0.05$.

to 2.33% and from 0.71% to 1.25%, respectively. Sabinene was also detected in mandarin, orange and grapefruit oil but at lower quantities; 0.21%, 0.39%, and 0.71%, respectively. Linalool, represented less than 0.57% of the total GC peak area. Decanal occurred in all oil samples at <0.36%. Caryophyllene (0.43%) and nootkatone (0.36%) were detected only in grapefruit oil. Trace amounts of neral (0.04%), geranial (0.065%), and valencene (0.03%) were found solely in orange oil.

Table 1
Constituent volatiles of commercial mandarin, orange and grapefruit oils

Compound/oil	Mandarin	Orange	Grapefruit
α-Pinene	1.25	0.71	0.80
Sabinene	0.21	0.39	0.71
Myrcene	2.33	2.12	2.31
Octanal	0	0	0.40
Limonene	88.89	86.80	92.34
γ-Terpinene	4.76	0	0
β-Ocimene	0	0	0.17
Linalool	0.54	0.56	0.10
Decanal	0.10	0.26	0.33
Caryophyllene	0	0	0.43
Nootkatone	0	0	0.37

Quantity of an individual compound is expressed as a percent of total GC peak area.

3.5. Composition of peel volatiles of citrus fruit at different developmental and maturity stages

GS–MS analysis of volatiles present in the peel of various citrus fruit cultivars at different developmental and maturity stages was performed to identify the major components released following wounding of peel tissue. The results confirm the data presented in Table 1 and clearly indicated that limonene is the major fruit peel volatile (Table 2). Its percentage ranged from 89% to 95% at the early stages of fruit development and the beginning of the harvest season (August–October). Limonene content, however, markedly decreased in clementine and grapefruit towards the end of the harvest season in clementine and grapefruit (41.92% and 60.33%, respectively). The percentage of the other detected volatiles was comparatively low at all developmental and maturity stages.

Analysis of the headspace of wounded fruit is shown in Table 3. The major group of chemicals found in wound headspace of clementine, orange and grapefruit were monoterpenes. Limonene, which composed 43.66–85.9% of the total GC peak area, was the most abundant followed by β-ocimene (9.44–18.29% in clementine and grapefruit, respectively), myrcene (1.31–5.73%), and α-pinene (0.05–3.19%). The most abundant monoterpenoids were linalool (0.18–1.52%)

Table 2
Seasonal changes in the concentration of constituent volatiles obtained from of clementine, orange and grapefruit

Compound	August 2004	October 2004	November 2004	December 2004	January 2004	February 2004	March 2004
Clemantine							
α-Pinene	0.57 ± 0.03	0.32 ± 0.16	0.33 ± 0.02	0.41 ± 0.03	0.11 ± 0.07	–	–
Sabinene	0.61 ± 0.09	0.57 ± 0.03	0.28 ± 0.02	0.65 ± 0.13	0.27 ± 0.18	–	–
Myrcene	1.69 ± 0.10	1.89 ± 0.23	1.41 ± 0.16	2.06 ± 0.07	0.81 ± 0.19	–	–
Limonene	93.77 ± 1.13	89.14 ± 1.33	74.62 ± 8.81	86.06 ± 1.25	41.92 ± 13.08	–	–
Linalool	1.11 ± 0.30	1.14 ± 0.35	1.20 ± 0.12	0.53 ± 0.01	0.25 ± 0.19	–	–
α-Terpineol	0.25 ± 0.13	0.50 ± 0.15	0.49 ± 0.06	0.40 ± 0.03	0.08 ± 0.08	–	–
Orange							
α-Pinene	0.62 ± 0.00	0.59 ± 0.02	0.38 ± 0.03	0.27 ± 0.09	0.55 ± 0.04	0.67 ± 0.04	0.70 ± 0.14
Sabinene	0.62 ± 0.10	0.62 ± 0.11	0.50 ± 0.12	0.27 ± 0.03	0.46 ± 0.09	0.74 ± 0.13	0.60 ± 0.09
Myrcene	1.93 ± 0.02	2.20 ± 0.02	1.61 ± 0.15	1.88 ± 0.13	1.91 ± 0.14	2.19 ± 0.06	2.21 ± 0.28
Limonene	94.77 ± 0.14	91.00 ± 0.25	74.86 ± 9.44	80.92 ± 2.65	72.41 ± 5.69	88.65 ± 0.21	81.77 ± 7.46
Linalool	0.48 ± 0.08	0.53 ± 0.20	0.91 ± 0.20	1.92 ± 0.36	0.83 ± 0.13	1.25 ± 0.06	0.58 ± 0.19
α-Terpineol	0.12 ± 0.01	0.25 ± 0.01	0.21 ± 0.06	0.68 ± 0.12	0.22 ± 0.04	0.33 ± 0.01	0.22 ± 0.01
Citral	0.30 ± 0.02	0.44 ± 0.07	0.29 ± 0.02	0.55 ± 0.13	0.24 ± 0.05	0.28 ± 0.01	0.13 ± 0.02
Red grapefruit							
α-Pinene	0.54 ± 0.02	0.59 ± 0.10	0.49 ± 0.03	0.39 ± 0.04	0.45 ± 0.03	0.33 ± 0.07	0.31 ± 0.04
Sabinene	0.29 ± 0.03	0.65 ± 0.16	0.22 ± 0.02	0.23 ± 0.01	0.21 ± 0.12	0.32 ± 0.08	0.10 ± 0.03
Myrcene	1.70 ± 0.01	2.14 ± 0.13	2.00 ± 0.06	2.40 ± 0.18	1.72 ± 0.06	1.01 ± 0.40	1.35 ± 0.13
Limonene	95.03 ± 0.14	90.17 ± 0.16	88.90 ± 0.87	69.78 ± 3.60	68.57 ± 1.07	60.63 ± 6.11	60.33 ± 2.98
Linalool	0.14 ± 0.02	0.24 ± 0.04	0.23 ± 0.02	0.35 ± 0.07	0.15 ± 0.01	0.22 ± 0.03	0.17 ± 0.01
α-Terpineol	0.11 ± 0.00	0.27 ± 0.03	0.23 ± 0.01	0.31 ± 0.01	0.19 ± 0.02	0.39 ± 0.08	0.22 ± 0.02
Citral	0.10 ± 0.01	0.30 ± 0.02	0.15 ± 0.02	0.26 ± 0.03	0.14 ± 0.01	0.23 ± 0.03	0.07 ± 0.01
Nootkatone	0.00	0.00	0.01 ± 0.01	0.86 ± 0.13	0.88 ± 0.18	1.36 ± 0.44	0.63 ± 0.28

Values are expressed as a percent of mean of total GC peak area ±S.E. of three samples taken from three different fruits.

and α-terpinol (0.65%, detected only in orange). The major sesquiterpenes detected were valencene (1.0 4–2.43% in orange and grapefruit, respectively), caryophyllene (0.68–1.05% in orange and grapefruit, respectively) and α copaene (<0.43%). The sesquiterpenoid, nootkatone (0.77%), was detected only in grapefruit headspace. The amount of the aliphatic aldehydes, nonanal, octanal, and decanal was 0.044%, 0.91% and 0.078%, respectively.

Table 3
Concentration of constituent volatiles from the dynamic headspace of wounded clementine, orange and grapefruit

Compound/oil	Clementine	Orange	Grapefruit
α-Pinene	3.19	3.05	0.05
Sabinene	0.74	0.80	0
Myrcene	5.73	4.22	1.31
Limonene	85.9	71.39	43.66
β-Ocimene	9.44	0	18.29
Linalool	0.18	1.52	0.91
Nonanal	0.04	0	0
Octanal	0	0.91	0
α-Terpeneol	0	0.65	0.09
Caryophyllene	0	0.68	1.04
Valencene	0	1.04	2.43
Limonene oxide	0	0	0.98
Decanal	0	0	0.07
α-Copaene	0	0	0.25
Nootkatone	0	0	0.77

Values are expressed as a percent of mean of total GC peak area of three samples taken from headspace of three different fruits.

3.6. Effect of monoterpene citrus volatiles on germination and growth of P. digitatum, P. italicum, P. expansum and B. cinerea

The effect of limonene, myrcene, α-pinene, and β-pinene on germination and germ tube growth of *P. digitatum*, *P. italicum*, *P. expansum* and *B. cinerea* is presented in Fig. 4. In general, the four monoterpenes were stimulatory to *P.* digitatum and *P.* italicum but inhibitory to or had no effect on *P. expansum* and *B. cinerea*. Germ tube elongation in *P. digitatum* responded most strongly to limonene and less strongly to α-pinene and β-pinene while myrcene had little effect. Germination of *P. digitatum* was stimulated by all of the test compounds. In *P. italicum*, myrcene stimulated germ tube elongation the most followed by limonene, with α-pinene, and β-pinene being about equal. Germination of *P. italicum* condia was highest in response to myrecene with the effect of the other compounds being about equal at concentrations of 5 μL or more per plate. Germ tube elongation in *P. expansum* was inhibited by all test compounds, with limonene and β-pinene also inhibiting germination. Germ tube growth and germination in *B. cinerea* were both greatly inhibited by all the test compounds, with β-pinene being the most potent compound.

3.7. Effect of oxidized citrus volatiles on germination and growth of P. digitatum, P. italicum, P. expansum, and B. cinerea

The effect of the monoterpene aldehyde, citral (a mixture of neral and geranial) and the monoterpene alcohol, linalool,

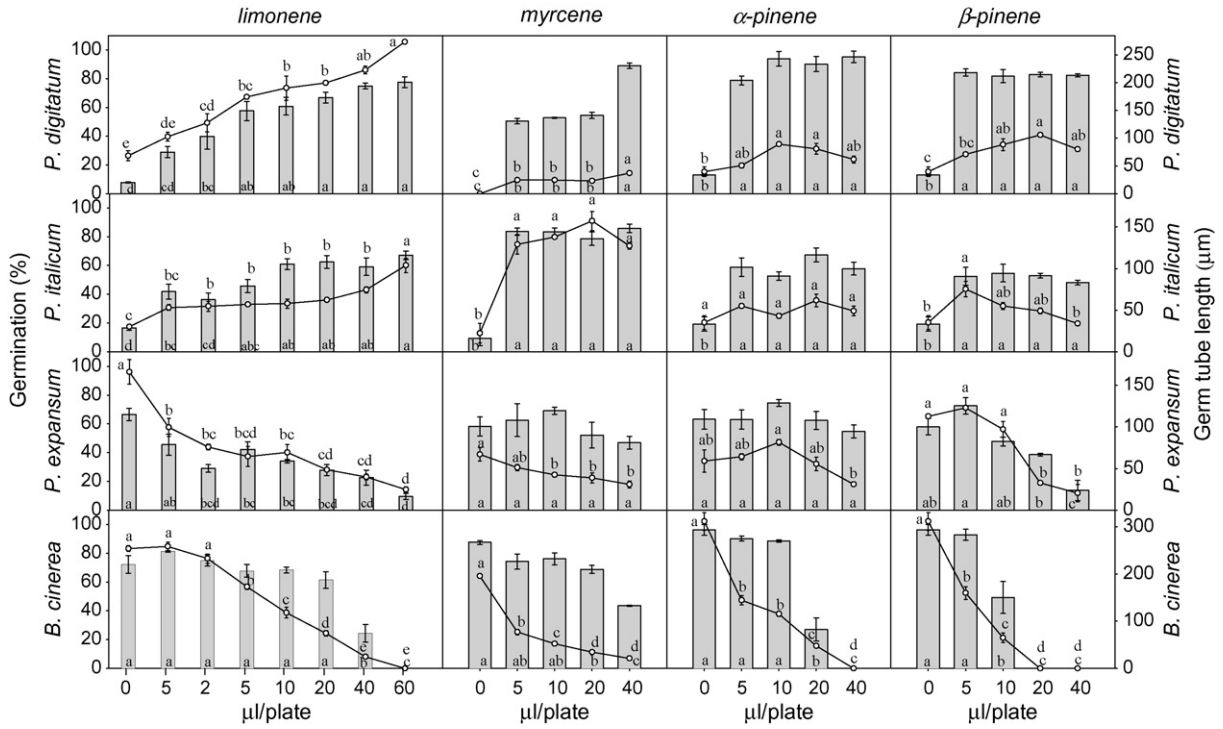

Fig. 4. Effect of the monoterpenes, limonene, myrcene, α-pinene, and β-pinene on percent germination ( ) and growth (-◯-) of *P. digitatum*, *P. italicum*, *P. expansum* and *B. cinerea*. Mean ± S.E. in three microscopic fields containing at least 30 spores each. Treatments with different letters are significantly different at $P \leq 0.05$.

on spore germination and germ tube growth of *P. digitatum*, *P. italicum*, *P. expansum*, and *B. cinerea* was very much concentration dependent (Fig. 5). The compounds were stimulatory at relatively low concentrations but strongly inhibitory at concentrations that were stimulatory for the other volatiles tested. Citral, exhibited a stimulatory effect in *P. digitatum* at 0.005 and 0.01 μL/plate and completely inhibited both germination and germ tube growth above those concentrations. In *P. italicum,* germination and growth were stimulated at concentrations up to 0.1 μL/plate but inhibitory at higher concentrations. Citral had very little effect on *P. expansum* or *B. cinerea* at concentrations up to 0.1 μL/plate but became completely inhibitory at concentrations higher than 0.5 μL/plate. In fact, all fungi were completely inhibited by citral at 1.0 5 μL/plate. Compared to citral, linalool significantly stimulated germination of *P. digitatum* and *P. italicum* at higher amounts (up to 2 μL/plate). The inhibitory effect of linalool on *P. expansum* and *B. cinerea* was evident at concentrations of 0.5 μL/plate and higher. Like citral and linalool, very low concentrations of the aliphatic aldehydes, octanal and nonanal, stimulated germ tube growth in *P. digitatum* and *P. italicum*, however, at higher concentrations (5 μL/plate), both compounds were inhibitory. While both compounds stimulated germination of *P. italicum*, they had little effect on the germination of *P. digitatum* (Fig. 6) *P. expansum* and *B. cinerea* were largely unaffected by <0.1 μL per plate of either compound but 0.5 μL per plate completely inhibited *P. expansum* germination and significantly inhibited germination in *B. cinerea*.

3.8. Germination of P. digitatum spores on peel of wounded and intact fruit

The effect of volatiles released from the oil glands of citrus fruit on the germination of *P. digitatum* spores was observed using fluorescent microscopy. Observations (micrographs not shown) clearly illustrate that fungal spores placed on the surface of lemon fruit, adjacent to a wounded oil gland, were induced to germinate and grow. The vast majority of the spores germinated and formed long germ tubes within 40 h after incubation even though they were not in contact with the oils that may or may not have leaked from the punctured glands. Massive growth of the fungus and further colonization of the wounded oil glands were observed after 140 h. While spores placed on an intact fruit surface did not germinate or grow even after 140 h of incubation.

4. Discussion

This research demonstrates that citrus fruit volatiles play an important role in host recognition by *P. digitatum* and *P. italicum*. Spores of both pathogens showed a 4–10-fold increase in percentage germination on water agar with exposure to volatiles emanating from fruit peel tissue of clementine, lemon, red grapefruit, white grapefruit, and orange, while volatiles derived from non-host fruit and vegetables (apple, pear, tomato, pepper, strawberry and avocado) had no effect. These data are in agreement with earlier reports describing the stimulatory effect of wound-released volatiles on germination of *P. digitatum* on water agar

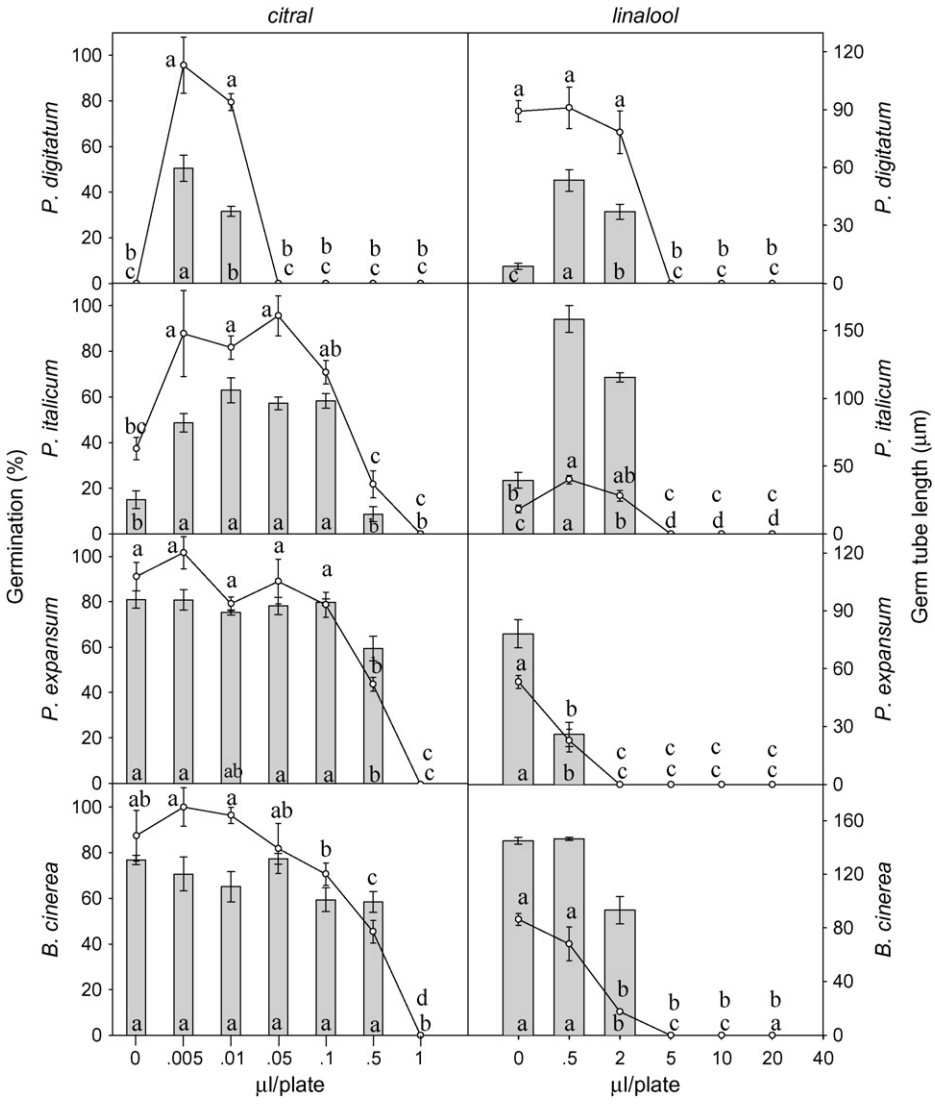

Fig. 5. Effect of the monoterpenoids, citral and linalool on percent germination ( ) and growth (-◯-) of *P. digitatum*, *P. italicum*, *P. expansum* and *B. cinerea*. Mean ± S.E. in three microscopic fields containing at least 30 spores each. Treatments with different letters are significantly different at $P \leq 0.05$.

(Eckert, 1992, 1994). The phenomenon of selective stimulation of fruit pathogens by volatile compounds is known in other fruit-pathogen systems. Volatile materials from the flesh of apples and nectarines stimulated spore germination of *P. expansum* and *Monilinia fructicola* but not *P. digitatum* (Eckert and Ratnayake, 1992). The ability of apples to promote germination of the pathogens was attributed to ethylene. Archbold et al. (1999) and Fallik et al. (1998) reported that in *in vitro* bioassays the presence of strawberry and blackberry fruit adjacent to agar media inoculated with *B. cinerea* spores stimulated germ tube elongation within the first 24 h. Spore germination of *Fusarium solani* on legumes is largely dependent on certain flavonoids, including defense-related isoflavoniod phytoalexins. Pea flavonoids have been shown to selectively stimulate pea pathogens and bean flavonoids stimulate only bean pathotypes (Ruan et al., 1995).

To determine if citrus fruit volatiles have a specific stimulatory effect solely on *P. digitatum* and *P. italicum*, the response of non-citrus pathogens, *P. expansum* and *B. cinerea*, to citrus peel volatiles was also investigated. Results demonstrated that while peel disks volatiles of clementine, orange and grapefruit stimulated germination of *P. digitatum* and *P. italicum*, germination of *P. expansum* and *B. cinerea* was either not affected or inhibited. The specific stimulatory effect of fruit peel volatiles on citrus pathogens and inhibitory effect on non-pathogens indicate that the possible role of volatile compounds in the host selectivity of some postharvest pathogens. In the case of citrus fruit, these volatiles are released from ruptured oil glands following mechanical wounding, facilitating the infection process. Similarly, volatiles of commercial citrus oil preparations were also stimulatory to pathogens and inhibitory to non-pathogens (Fig. 4). In general, our results are in agreement with data originally presented by French et al. (1978) but differ regarding the optimal concentration of biologically active essential oils. French et al. (1978) reported that the germination of conidia of *P. digitatum* and *P. italicum* was stimulated by suspensions of orange oil in a range from 50 to 10,000 ppm, defining 250 ppm as the most effective. Our results indicate that oil concentrations between 60 and 150 ppm are the most effective for stimulation

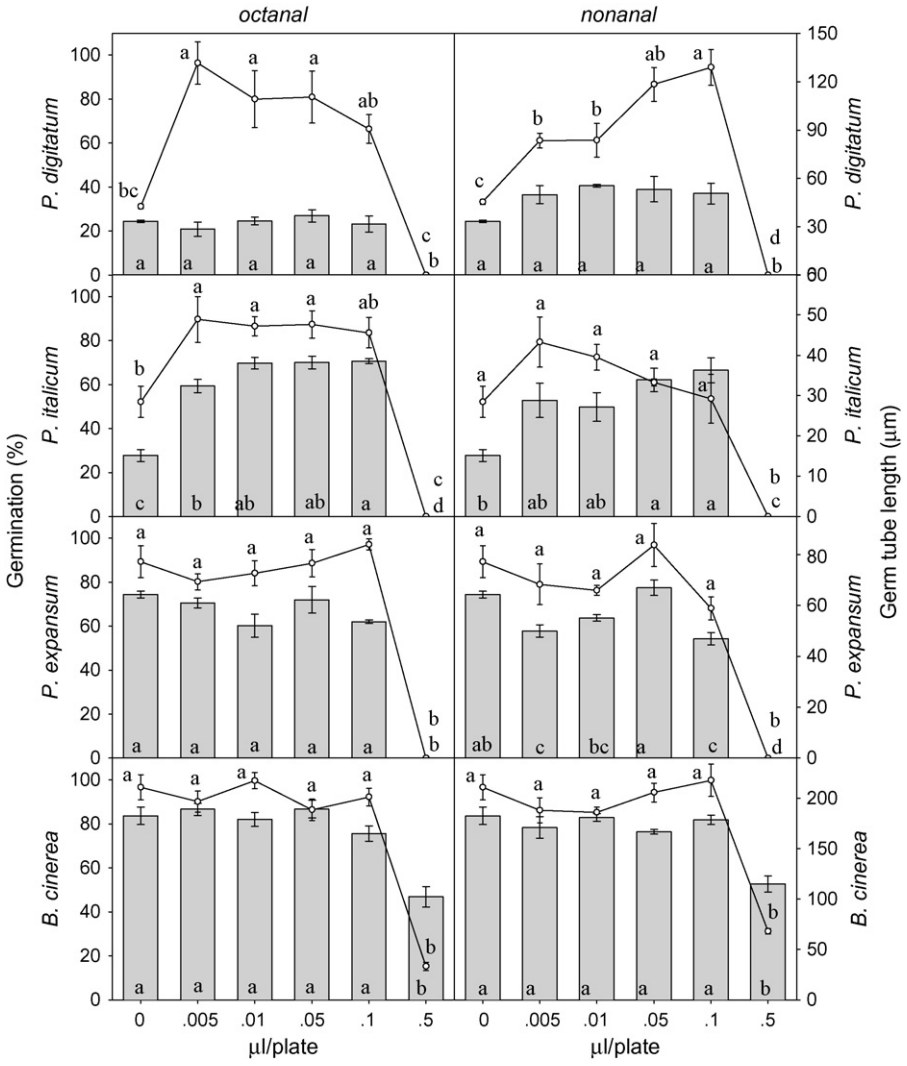

Fig. 6. Effect of the aliphatic aldehydes, octanal and nonanal on percent germination ( ) and growth (-◯-) of *P. digitatum*, *P. italicum*, *P. expansum* and *B. cinerea*. Mean ± S.E. in three microscopic fields containing at least 30 spores each. Treatments with different letters are significantly different at $P \leq 0.05$.

of germination of *P. digitatum* and *P. italicum*. This discrepancy could be due, however, to the differences in the experimental approaches used in the two studies. We applied variable quantities of an oil on a paper disc attached to the top of a Petri dish in order to create different concentrations of volatiles in the Petri dish headspace, while French et al. (1978), used water agar amended with orange oil creating an emulsion in which spores were in direct contact with the oil. The latter approach could result in a great deal of variability in the exposure of a spore to a specific oil concentration and thus may have underestimated the effect of concentration on spore germination.

We also demonstrated a strong, inhibitory effect of volatiles of citrus essential oils on *B. cinera* and *P. expansum* germination and germ tube growth at concentrations as low as 60 ppm but did not detect any significant inhibition of *P. digitatum* and *P. italicum* at concentrations up to 600 ppm. These results provide additional evidence that *P. digitatum* and *P. italicum* are uniquely adapted to and stimulated by the volatile environment associated with citrus wounds whereas this same environment is inhibitory to other non-citrus pathogens.

Despite the fact that the stimulatory effect of citrus oils has been previously documented (French et al., 1978) no attempt was made in that report to determine the role of individual constituents of citrus peel volatiles. Although, the qualitative and quantitative composition of citrus essential oils and peel extracts is well investigated (Vekiari et al., 2002; Umano et al., 2002; Njoroge et al., 2005) the data regarding the volatile components of the atmosphere surrounding wounded citrus fruit are incomplete and conflicting (Norman et al., 1967; Norman, 1977; Eckert et al., 1994). We performed a comparative CG–MS quantification and identification of volatile constituents of citrus commercial oils (Table 2), peel extracts (Table 1) and the headspace of the wounded fruit (Fig. 5). Monoterpene hydrocarbons (limonene, α-pinene, sabinene, and myrcene) were the most abundant in all volatiles regardless of the source. Among sesquiterpenes, valencene and cariophyllene were also detected. The aliphatic aldehyde, decanal, occurred in all oils and peel extracts but was not detected in the wound headspace of orange. Linalool (alcohol) was also found in all samples subjected to analysis. Oxygenated compounds, such as neral and geranial,

were not identified in the atmosphere surrounding wounded fruit and detected only in peel extracts of orange and grapefruit, and trace amounts in orange oil. Nootkatone was uniquely detected in grapefruit oil, grapefruit peel extract and the headspace of wounded grapefruit. This compound is known to be specific to grapefruit and is responsible for the unique aroma of the fruit (MacLeod, 1967; Del Rio et al., 1992). Stange et al., 2002 suggested that nootkatone plays a role in enhancing the susceptibility of grapefruit to *Penicillium* rots.

Considerable effort was made to identify the wound volatiles of citrus fruit and determine the biological role of the most abundant ones. French et al. (1978) reported that citral, nonanal, and citronella applied as volatiles stimulated the germination of *P. italicum* at concentration of 100, 50 and 100 ppm, respectively, while *P. digitatum* was only stimulated by citral and nonanal at 500 ppm. Eckert and Ratnayake (1994) reported that combinations of limonene, acetaldehyde, ethanol and carbon dioxide stimulated spore germination of *P. digitatum* but that each individual component alone had no effect. Aliphatic aldehydes (C-9) were found to inhibit germination of *P. digitatum* conidia; octanal was the most potent, with an IC-50 of 60 ppm (Davis and Smoot, 1972). Citral has been shown to reduce germ tube elongation of *P. digitatum*, with an IC-50 of ~100 ppm (Rodov et al., 1995). We investigated the effect of the most abundant volatile compounds present in different citrus fruit, especially those present in the fruit wound headspace. Our results clearly demonstrated stimulation of germination and germ tube growth in both *P. digitatum* and *P. italicum* by limonene, myrcene, α-pinene, and β-pinene (Fig. 6). Limonene was shown to be the most efficient in induction of germination and growth in both pathogens. The stimulatory effect was concentration dependent but even at low concentrations (≤6 ppm) a significant stimulation was observed. Myrcene, α-pinene, and β-pinene also substantially enhanced germination and growth but at higher concentrations (≤60 ppm). At similar concentrations, all of these monoterpenes were inhibitory to *P. expansum* and *B. cinerea*. Thus, our findings conflict with the earlier report by Eckert and Ratnayake (1994) in which individual monoterpenes (limonene, myrcene, α-pinene,) were not stimulatory to either *P. digitatum* and *P. italicum* when tested on water agar. These conflicting findings may again be the result of the use of different assay systems and argue for the need to have standard protocols when conducting volatile research on host pathogen interactions.

Oxygenated volatiles of the citrus peel have been reported to have both stimulatory (French et al., 1978) and inhibitory (Rodov et al., 1995) effects on *P. digitatum* and *P. italicum*. In the present study, tests performed with citral (a mixture of neral and geranial) and linalool indicated that their biological activity is strongly dependent on the concentration of the volatile. Citral strongly stimulated germination of *P. digitatum* at concentrations equivalent to 0.06–0.15 ppm but completely inhibited germination at higher concentrations. *P. italicum* was stimulated over a wider range of concentrations (from 0.06 to 1.5 ppm) and complete inhibition was not evident until 15 ppm. In general, citral showed only slight inhibitory activity against *P. expansum* and *Botrytis* at concentrations less than 6 ppm, however, completely arrested fungal development at 15 ppm. Antifungal activity has been attributed to citral against *P. digitatum* and *P. italicum* and it has been suggested that it is part of a preformed defense mechanism in citrus fruit (Rodov et al., 1995). Despite this report, however, our results demonstrated that citral concentrations are under the detection limit in the atmosphere surrounding wounded fruit. Therefore, we assume that its involvement in either stimulation or inhibition of citrus pathogens is negligible.

The monoterpenes, limonene, together with pinene and myrcene, are the predominant volatile chemicals present in the atmosphere surrounding wounded fruit in several different citrus species. These compounds along with other minor ones were shown to promote germination of conidia of *P. digitatum* and *P. italicum* in the absence of nutrients both on water agar and on the fruit surface (Fig. 9), and exerted a significant inhibitory effect against non-citrus pathogens (*P. expansum* and *B. cinerea*). We suggest that these monoterpene volatiles, particularly limonene, serve as the main signaling molecules in host recognition by *P. digitatum* and *P. italicum*. However, the role of the other minor components of the oil cannot be excluded. While it is reasonable to suggest that all the component volatiles emanating from wounded citrus fruit may have a role in the stimulation and growth of *P. digitatum* and *P. italicum*, the relative importance of each component separately is not clear. In the present study, we demonstrated that most of these components individually can promote germination and growth but that the most effective concentration varies according to the compound. Little is known how optimum concentrations, and activity in general, may be affected (additively, synergistically, or negatively), however, by combinations of the compounds. Previous reports (French et al., 1978; Eckert and Ratnayake, 1994) showed synergistic effects of more than one volatile component. Although this effect may exist, our findings indicate that it is hard to conclusively prove this claim in an *in vivo* situation. A more basic, biochemical and molecular understanding of how these chemicals trigger germination in *P. digitatum* and *P. italicum* is needed.

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